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Control of the Shift from Homolactic Acid to Mixed-Acid Fermentation in *Lactococcus lactis*: Predominant Role of the NADH/NAD$^+$ Ratio

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During batch growth of *Lactococcus lactis* subsp. *lactis* NCDO 2118 on various sugars, the shift from homolactic to mixed-acid metabolism was directly dependent on the sugar consumption rate. This orientation of pyruvate metabolism was related to the flux-controlling activity of glyceraldehyde-3-phosphate dehydrogenase under conditions of high glycolytic flux on glucose due to the NADH/NAD$^+$ ratio. The flux limitation at the level of glyceraldehyde-3-phosphate dehydrogenase led to an increase in the pool concentrations of both glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate and inhibition of pyruvate formate lyase activity. Under such conditions, metabolism was homolactic. Lactose and to a lesser extent galactose supported less rapid growth, with a diminished flux through glycolysis, and a lower NADH/NAD$^+$ ratio. Under such conditions, the major pathway bottleneck was most probably at the level of sugar transport rather than glyceraldehyde-3-phosphate dehydrogenase. Consequently, the pool concentrations of phosphorylated glycolytic intermediates upstream of glyceraldehyde-3-phosphate dehydrogenase decreased. However, the intracellular concentration of fructose-1,6-bisphosphate remained sufficiently high to ensure full activation of lactate dehydrogenase and had no in vivo role in controlling pyruvate metabolism, contrary to the generally accepted opinion. Regulation of pyruvate formate lyase activity by triose phosphates was relaxed, and mixed-acid fermentation occurred (no significant production of lactate on lactose) due mostly to the strong inhibition of lactate dehydrogenase by the in vivo NADH/NAD$^+$ ratio.

The industrial importance of lactic acid bacteria is based on their ability to rapidly ferment sugars into lactic acid. For example, metabolism in the homolactic acid bacteria (the model organism is *Lactococcus lactis*) leads to >90% conversion of sugars to lactic acid. However, under certain conditions, this homolactic behavior is lost and increased amounts of other metabolites, such as formate or CO_2, acetate, and ethanol, are produced in what is generally called mixed-acid fermentation. This behavior was first observed in glucose-limited chemostat cultures (22). Homolactic behavior was seen only during rapid growth in which significant amounts of glucose remained in the medium; mixed-acid fermentation was observed at lower rates of growth and true carbon-limited chemostat steady states. Such a mixed metabolism may also occur under carbon-excess conditions with certain sugars. Galactose metabolism of *L. lactis* results in a fermentation profile in which significant amounts of acetate and ethanol are produced (23), though lactic acid remains the major product (60% of the galactose consumed). A less pronounced shift toward mixed-acid metabolism is also observed during growth on maltose (11, 18). Although details of the biochemical pathways involved remain obscure, the use of pentose sugars involves significant acetate synthesis (9). Under conditions of carbon excess, sugar metabolism in *L. lactis* often appears to be dependent on the metabolic pathways involved (Fig. 1). A rapid and homolactic metabolism is generally associated with the tagatose pathway, while mixed-acid fermentation occurs when the Leloir pathway is functional during growth on either lactose or galactose (6, 25). However, this correlation is an oversimplification since many strains of *L. lactis* retain a mixed-acid fermentation in the absence of the Leloir pathway or a homolactic profile of fermentation when the two pathways are believed to be operative (23, 24).

An alternative interpretation would be that rapid flux through the central pathways is likely to result in homolactic fermentation while diminished rates of sugar metabolism will provoke a shift toward a mixed-acid fermentation, thus providing a common basis for explaining both chemostat and batch culture data. Such a shift involves a modification of pyruvate metabolism with a decreased activity of lactate dehydrogenase (LDH) and an increase in pyruvate formate lyase (PFL) (anaerobic conditions) or pyruvate dehydrogenase (aerobic conditions) activity. Inhibition of the PFL by glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phosphate (DHAP) associated with activation of the LDH by fructose-1,6-bisphosphate (FBP), all metabolites shown to be present at significantly higher concentrations during homolactic metabolism, has been claimed to control this shift of metabolism (22, 23, 27). The frequent use of nonproliferating cells rather than actively fermenting cells has made extrapolation of these intracellular concentrations to in vivo regulation mechanisms difficult. In addition, no biochemical basis for the variation in metabolite pools has been proposed, though Poolman et al. (14) have suggested that the enzyme GAP dehydrogenase may have a controlling influence during homolactic fermentations.

To better understand the shift from homolactic to mixed-acid metabolism, we used *L. lactis* subsp. *lactis* NCDO 2118,

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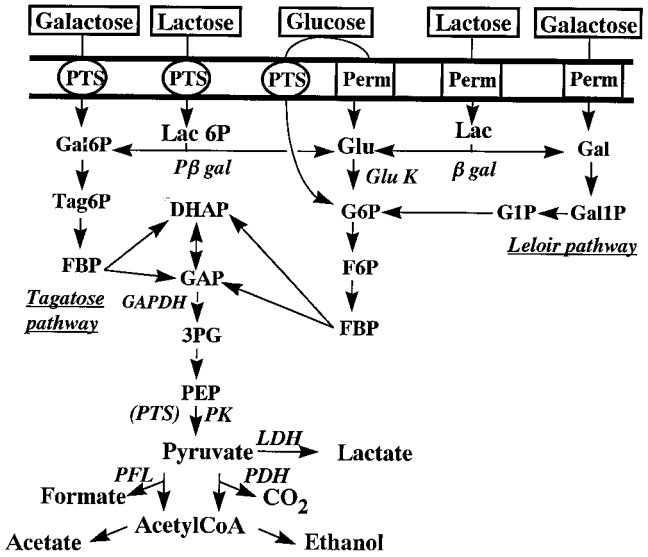

FIG. 1. Metabolic pathways involved in the uptake and metabolism of glucose, galactose, and lactose by *L. lactis*. Perm, permease-mediated sugar uptake; Gal6P, galactose-6-phosphate; Lac 6P, lactose-6-phosphate; Tag6P, tagatose-6-phosphate; TBP, tagatose bisphosphate; *Pβ gal*, phospho-β-galactosidase; β *gal*, β-galactosidase; *GAPDH*, GAP dehydrogenase; *PK*, pyruvate kinase; *PDH*, pyruvate dehydrogenase. Other abbreviations are defined in the text.

which has the capacity to grow on simple defined medium (2). This bacterium, isolated from vegetal matter, lacks the plasmid-encoded alternative pathways linked to rapid growth in milk such as the lactose plasmid, containing the genes of the lactose phosphotransferase transport system (PTS), phospho-β-galactosidase, and enzymes of the tagatose pathway (5), or the citrate permease plasmid (15). Recently, the growth rate of this strain has been examined on a range of carbon substrates, and the fermentation profile has been analyzed (3). Homolactic metabolism occurs on substrates supporting rapid growth, and a mixed-acid fermentation occurs on those supporting slow growth. The simplified metabolic architecture of this strain facilitates the physiological characterization, and the data obtained in the present study demonstrate clearly that pathway flux is the major factor determining the metabolic behavior of this strain. Though the concentrations of metabolic intermediates clearly influence pyruvate metabolism, the major phenomenon involved is the ratio of NADH/NAD^+, which controls carbon flux through both the GAP dehydrogenase and LDH. This additional information together with a detailed analysis of metabolite concentrations and enzyme kinetic characterization modifies and significantly extends our understanding of the metabolic behavior of *L. lactis.*

MATERIALS AND METHODS

Organism and growth conditions. The bacterium used throughout this work was *L. lactis* subsp. *lactis* NCDO 2118, which was grown on MS10 medium (2) with various carbon substrates at the same concentration (330 mM carbon).

Cultures were grown under anaerobic conditions in butyl-rubber-stoppered tubes or in a 2-liter fermentor (Setric Genie Industriel, Toulouse, France) at a temperature of 30°C, pH of 6.6, and agitation speed of 250 rpm. Cultures in the fermentor were maintained at pH 6.6 by automatic addition of KOH (10 N). Inoculation was at 2% with exponential-phase cells from precultures grown on the same medium.

Analytical methods. Bacterial growth was monitored spectrophotometrically at 580 nm and calibrated against cell dry weight measurements. A change of 1 U of density was shown to be equivalent to 0.31 g of dry matter per liter, irrespective of the carbon substrate used. Sugars and fermentation products (lactate, formate, acetate, and ethanol) were determined by high-pressure liquid chromatography as previously described (2).

Preparation of crude extract. A volume of culture corresponding to 100 mg (dry weight) of cells was centrifuged (4°C, 10 min at 6,000 × *g*) and washed twice with 0.2% (vol/vol) KCl. Cells were resuspended in Tris (45 mM)-tricarballylate (15 mM) buffer (pH 7.2) containing glycerol (20%), $MgCl_2$ (4.5 mM), and dithiothreitol (1 mM). Cell disruption by sonication (five cycles of 30 s interspaced by 1-min cooling periods) was followed by the removal of cell debris by centrifugation for 10 min at 6,000 × *g* and 4°C. The supernatant was used for all enzyme assays (except the PFL activity).

Cell extracts for assaying PFL activity were prepared in an anaerobic chamber maintained under N_2-H_2-CO_2 (80%:10%:10%) gas phase. A volume of culture corresponding to 40 mg (dry weight) of cells was centrifuged (4°C, 10 min at 6,000 × *g*) and washed twice with potassium phosphate buffer (50 mM, pH 7.2) containing dithiothreitol (2 mM). Bacteria were disrupted by sonication as described above. The resulting crude extract was centrifuged (4°C, 10 min at 6,000 × *g*), and the supernatant was used for PFL assays.

The protein concentration of enzymatic extracts was determined by the Lowry method (13), with bovine serum albumin as the standard.

Enzyme assays. All enzymes were assayed immediately after cell disruption at 30°C and pH 7.2. Spectrophotometric measurement of the level of NADH or 2-(*p*-iodophenyl)-3-*p*-nitrophenyl tetrazolium chloride (INT; an artificial electron acceptor) was monitored at 340 nm ($\varepsilon = 6.22.10^3\ M^{-1}\ cm^{-1}$) or 500 nm ($\varepsilon = 12.4.10^4\ M^{-1}\ cm^{-1}$), respectively. One unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of product per minute.

GAP dehydrogenase was assayed in a reaction mixture containing triethanolamine-HCl buffer (125 mM, pH 7.2), NAD^+ (1 mM), sodium arsenate (5 mM), cysteine-HCl (5 mM), and GAP (2 mM). Assays were initiated by the addition of GAP. No detectable activity of NADPH-dependent GAP dehydrogenase could be measured.

LDH was measured by using a slightly modified form of the assay procedure of Cocaign-Bousquet and Lindley (4). The reaction mixture contained Tris-HCl buffer (100 mM, pH 7.2), $MgCl_2$ (5 mM), NADH (0.3 mM), FBP (3 mM), and sodium pyruvate (20 mM), which was used to initiate the reaction.

Pyruvate kinase was assayed by the method described by Thomas (20), with an optimized reaction mixture consisting of Tris-HCl buffer (100 mM, pH 7.2), $MnSO_4$ (5 mM), KCl (10 mM), ADP (1 mM), NADH (0.3 mM), LDH (10 U), and phosphoenolpyruvate (PEP; 2 mM). As glucose-6-phosphate (G6P; 3 mM) was a stronger activator than FBP, it was added in the assay. The reaction was initiated by the addition of PEP.

Pyruvate dehydrogenase was assayed by the method described by Cocaign-Bousquet and Lindley (4), in a medium containing phosphate buffer (100 mM, pH 7.2), $MgCl_2$ (5 mM), INT (0.6 mM), bovine serum albumin (1 g/liter), lipoamide dehydrogenase (0.1 mg/liter), dithiothreitol (0.3 mM), coenzyme A (0.2 mM), thiamine pyrophosphate (0.2 mM), NAD^+ (2 mM), and pyruvate (5 mM). The assay was initiated by the addition of pyruvate. No activity could be detected in cells grown under anaerobic conditions, even though the assay system used detected activity in cells grown in microaerated cultures.

PFL activity measurements were undertaken in an anaerobic chamber as described by Takahashi et al. (19). The reaction mixture contained phosphate buffer (100 mM, pH 7.2), pyruvate (20 mM), coenzyme A (0.2 mM), NAD^+ (2 mM), dithiothreitol (1.5 mM), citrate synthase (2.5 U), and L-malate dehydrogenase (50 U). The assay was initiated by the addition of pyruvate. An alternative method based on the measurement of formate production, using NAD^+-dependent formate dehydrogenase, was also tested and gave identical results.

Estimation of intracellular metabolites and cellular coenzyme concentration. Intracellular metabolite and coenzyme concentrations were measured by using an in vitro procedure based on rapid inactivation of metabolism followed by metabolite extraction directly in the cell sample. Cells samples were removed from the culture, frozen immediately in liquid nitrogen, and stored at −20°C. Methods of metabolite extraction (acidic or basic) and assay procedures were based on those developed by Lebloas et al. (10). A variable volume of either HCl (36%) or KOH (10 N) was added during thawing to give a final pH of 1.2 or 12.5, respectively. The acid extraction procedure used for most metabolites and coenzymes was achieved by incubating the HCl-treated culture (pH 1.2) at 50°C for 8 min before neutralizing to pH 6.5 to 7 by KOH (10 N) while agitating vigorously. After centrifugation for 8 min (4°C and 12,000 rpm), the supernatant was immediately used for metabolite concentration measurements. Acid-labile coenzymes, like NADH, were extracted by incubating the KOH-treated culture (pH 12.5) for 10 min at room temperature (25°C). After centrifugation at 4°C for 8 min at 12,000 rpm, the supernatant was immediately tested for NADH without neutralizing to avoid destruction of NADH by locally high concentrations of acid.

Metabolites were measured by coupling appropriate enzyme assays with fluorimetric determination of the coenzyme NADH or NADPH. Emission was measured at 460 nm after excitation at 350 nm with a fluorescence spectrophotometer (Hitachi F-2000). G6P, fructose-6-phosphate (F6P), and glucose-1-phosphate (G1P) were measured by using a mixture which contained 200 μl of triethanolamine buffer (500 mM, pH 7.6, containing 15 mM $MgSO_4$ and 4 mM EDTA), 20 μl of NADP (10 mM), 400 μl of extract, 380 μl of H_2O, and 10 μl of G6P dehydrogenase (200 U/ml) to initiate G6P consumption. After the reaction was completed, 10 μl of phosphoglucose isomerase (200 U/ml) was added to measure the F6P concentration; after completion of the reaction, 10 μl of phosphoglucomutase (200 U/ml) was added to measure the G1P concentration. FBP, GAP, and DHAP concentrations were measured by using an assay mixture

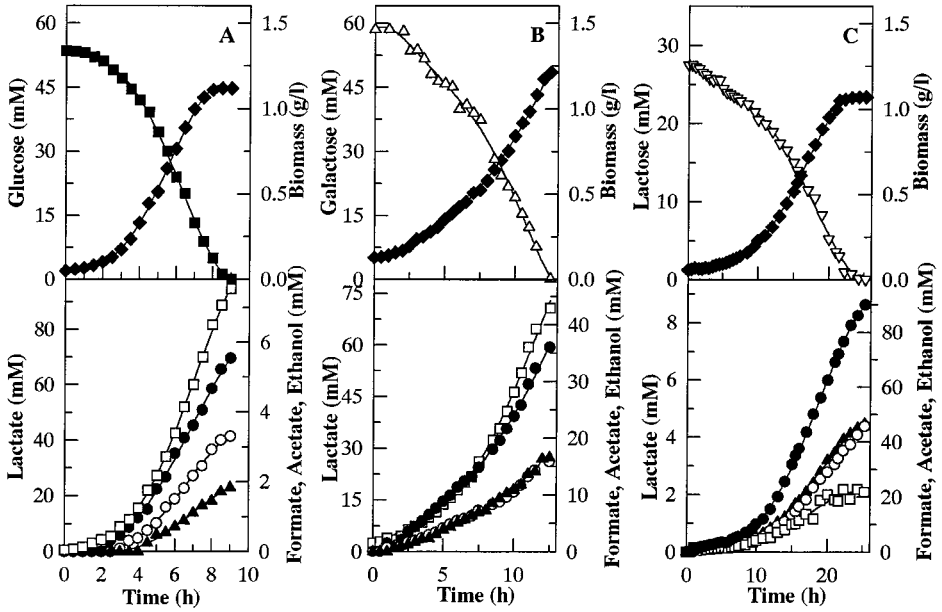

FIG. 2. Fermentation time course for *L. lactis* growing on glucose (A), galactose (B), or lactose (C) at 30°C and pH 6.6. Data points represent biomass (◆), glucose (■), galactose (△), lactose (▽), lactate (□), formate (●), acetate (○), and ethanol (▲).

containing 200 μl of triethanolamine buffer (see above), 20 μl of NADH (0.5 mM), 400 μl of extract, 380 μl of H_2O, and 10 μl of glycerophosphate dehydrogenase (500 U/ml) to initiate DHAP consumption. After complete depletion of the DHAP present in the extract, 10 μl of triose phosphate isomerase (500 U/ml) was added to measure the GAP concentration. Addition of 10 μl of fructose bisphosphate aldolase (200 U/ml) allowed the FBP concentration present in the extract to be measured. 3-Phosphoglycerate (3-PG) and 1,3-diphosphoglycerate (1,3-PG) were measured by using an assay mixture containing 200 μl of triethanolamine buffer (see above), 25 μl of NADH (0.5 mM), 20 μl of ATP (100 mM), 400 μl of extract, 355 μl of H_2O, and 20 μl of GAP dehydrogenase (500 U/ml) to initiate 1,3-PG consumption. After the reaction was completed, 20 μl of phosphoglycerate kinase (1,000 U/ml) was added to measure 3-PG concentration. PEP was determined after complete depletion of the pyruvate present in the extract by using a mixture which contained 200 μl of potassium phosphate buffer (500 mM, pH 7, containing 12.5 mM $MgCl_2$), 20 μl of ADP (100 mM), 40 μl of NADH (0.5 mM), 300 μl of extract, 440 μl of H_2O, and 20 μl of LDH (100 U/ml). After all the pyruvate had been consumed, 20 μl of pyruvate kinase (1,000 U/ml) was added to initiate PEP consumption.

NAD^+ was assayed by using 200 μl of pyrophosphate buffer (250 mM, pH 8.8, containing 12 g of semicarbazide per liter), 10 μl of absolute ethanol, 300 μl of extract, 490 μl of H_2O, and 10 μl of alcohol dehydrogenase (4 mg/ml). The NADH present in the alkaline extract was measured in a reaction mixture containing 200 μl of triethanolamine buffer (500 mM, pH 7, containing 15 mM $MgSO_4$ and 4 mM EDTA), 300 μl of alkaline extract, 480 μl of H_2O, 20 μl of pyruvate (200 mM), and 20 μl of LDH (1,000 U/ml) to initiate the pyruvate-dependent oxidation of NADH.

All metabolite concentrations were obtained relative to cell dry weight but were expressed as aqueous molar values, using the average intracellular volume of 1.7 ml/g reported by Sjoberg and Hahn-Hagerdal (16). Enzymatic determination of intracellular pyruvate was not possible due to significant interference with a medium component, but it can be concluded that the pyruvate pool remained low (<2 mM) since alternative detection methods using high-pressure liquid chromatography (Dionex) equipped with an AG11 column and conductimetric detection could not detect any peak for pyruvate (detection threshold, 1 mM).

RESULTS

Kinetic analysis of glucose, galactose, and lactose fermentations. Growth of *L. lactis* on lactose, glucose, or galactose was investigated in batch cultures in pH-regulated fermentors (Fig. 2). Glucose supported rapid growth (specific growth rate of 0.58 h^{-1}), while galactose or lactose supported significantly slower rates of growth (0.23 and 0.20 h^{-1}, respectively). Lactate, formate, acetate, and ethanol were produced throughout the cultures but in quantities dependent on the substrate being used. Carbon recovery was similar in the three fermentations and represented 90 to 92% of the analyzed products. Lactate was the major product (95 mM) of glucose-grown cells: the alternative products of pyruvate metabolism (acetate, formate, and ethanol) represented only 7% of recovered products. In the case of lactose, the production of formate, acetate, and ethanol accounted for the majority (96%) of the carbon recovered as fermentation end products, while virtually no lactate was produced (2 mM). Galactose-grown cells showed an intermediary product profile. The amount of formate produced was always in good agreement with the sum of acetate and ethanol production, indicating that PFL was the only alternative pathway of pyruvate metabolism; i.e., there was no flux through pyruvate dehydrogenase. The flux of pyruvate through PFL led to equimolar quantities of acetate and ethanol, thereby satisfying the energetic constraints of these fermentations.

Growth rates and fermentation product profiles confirm previous data (3) and show that homolactic fermentation is observed in media supporting high growth rates, while substrates supporting lowest growth rates (lactose and galactose) show a mixed-acid fermentation profile. However, galactose, which supported a growth rate similar to that supported by lactose,

TABLE 1. Specific rates of growth, sugar consumption, and product formation during batch growth of *L. lactis* on glucose, galactose, or lactose[a]

Growth on:	Specific rate of:					
	Growth (h^{-1})	Sugar consumption (mmol/g/h)	Product formation (mmol/g/h)			
			Lactate	Formate	Acetate	Ethanol
Glucose	0.58	19.4	32.0	2.0	0.7	0.8
Galactose	0.23	10.8	11.2	6.1	2.8	2.6
Lactose	0.20	7.2	0.5	11.3	5.0	5.6

[a] The rates given are maximal rates observed during the exponential growth phase. Sugar consumption rates are expressed as hexose equivalents.

TABLE 2. Glycolytic metabolite concentrations in cells harvested throughout the exponential growth phase of *L. lactis* on various sugars

Metabolite	Concn (mM)[a] in cells grown on:		
	Glucose	Galactose	Lactose
G6P	25 ± 3	25 ± 3	15 ± 2
F6P	1.8 ± 0.6	5.9 ± 0.5	2.9 ± 0.6
FBP	118 ± 15	36 ± 16	26 ± 9
DHAP	21 ± 3	<0.6	<0.6
GAP	6 ± 2	<0.6	<0.6
1,3-PG	<0.6	<0.6	<0.6
3-PG	<0.6	<0.6	<0.6
PEP	25 ± 10	<0.6	<0.6

[a] Average of at least four samples taken during the exponential growth phase.

produced significantly more lactate. Comparison of the specific rates of sugar consumption (Table 1) showed that the metabolic shift can be closely correlated to the glycolytic flux. Thus, while growth rates were similar during fermentation of lactose or galactose, the extent to which pyruvate is oriented through PFL is inversely proportional to the sugar consumption rate (i.e., flux through glycolysis). The lack of direct correlation between growth rate and sugar consumption rate is due to the additional gain in ATP from acetate synthesis. Thus, growth on lactose is almost as high as that on galactose due to the increased acetate synthesis during lactose metabolism and the influence that this has on ATP production (2.68 mol of ATP/mol of hexose for lactose and 2.10 mol of ATP/mol of hexose for galactose).

Biochemical analysis of glucose, galactose, and lactose fermentations. (i) Specific activities of glycolytic enzymes. Various enzymes of glycolysis were measured in cells taken from the exponential phase of cultures grown on glucose, galactose, or lactose. Pyruvate kinase was present at similar concentrations on all three substrates, while others were expressed to different levels on each substrate. This was true for LDH, which was present at twofold-higher levels on glucose than on either galactose or lactose, and for PFL, whose activity was twofold lower on glucose than on the other substrates. However, these modified concentrations of active enzymes do not allow the metabolic shift to be explained in comparison to the changes in flux through these enzymes, since the flux through LDH decreased 66 times and the flux through PFL increased about 6 times from glucose to lactose consumption.

GAP dehydrogenase is the only glycolytic enzyme producing NADH and thus contributes to the fixed stoichiometry of sugar fermentation in *L. lactis*, for which pyruvate metabolism is the only means to ensure reoxidation of NADH. Furthermore, this enzyme has been postulated to have a controlling influence on glycolysis (15). Specific activity of GAP dehydrogenase showed some variation, being somewhat higher during growth on galactose (1,800 nmol/min/mg) than on either glucose or lactose (1,200 nmol/min/mg), but once again, the modified levels of gene expression cannot alone explain the different fermentation patterns obtained since these modifications are not proportional to pathway flux.

(ii) Metabolite pool concentrations. The concentrations of phosphorylated glycolytic intermediates were measured in exponentially growing cells taken from each fermentation and are summarized in Table 2. FBP, considered the major activator of LDH (21), was the metabolite found in highest concentrations within the cell. This was true for all substrates, but the concentration measured in glucose-grown cells (118 mM) was at least threefold higher than that in cells grown on either galactose or lactose. GAP and DHAP, strong inhibitors of PFL (19, 23), were also present at high concentrations on glucose (21 and 6 mM, respectively), though intracellular concentrations below the assay precision threshold were measured on both galactose and lactose (<0.6 mM). The ratio of NADH/NAD^+ was found to be directly correlated with the sugar consumption rate measured on the three substrates (Fig. 3, insert).

Kinetic parameters of key enzymes. The concentrations of key enzymes involved in pyruvate metabolism do not satisfactorily explain the shift from homolactic to mixed-acid fermentation; therefore, the possibility that these enzymes are subject to biochemical modulation by various glycolytic metabolites was examined.

(i) LDH. The affinity constant (K_m) of LDH for pyruvate was estimated to be 3 mM in crude extracts, and the maximum rate was 4,300 nmol/min/mg of protein. Although no LDH activity could be measured in the absence of FBP, maximum activation of LDH was attained with the addition of low concentrations of FBP (2 μM). Inorganic phosphate (P_i) has been cited as an inhibitor of LDH activity (26). This finding was confirmed here and was associated with an increase in the activation constant (K_{act}) for FBP. However, only a weak inhibitory effect was observed, as the K_{act} increased to 130 μM at P_i concentrations as high as 500 mM. Furthermore, no inhibitory effects of P_i (K_m or V_{max}) on LDH activity were observed when concentrations of P_i far exceeding the intracellular phosphate pool concentration (typically in the range 50 to 100 mM) were used in assay mixtures containing an FBP concentration ensuring full activation of the enzyme (6 mM in the assay). In light of this finding, it seems improbable that either the FBP or the phosphate pool plays any significant in vivo role in control of LDH activity. Furthermore, the inhibitory effect of PEP on LDH activity was low over the in vivo range of PEP concentrations; i.e., <5% inhibition was observed with 20 mM PEP. However, LDH was extremely sensitive to variations in the NADH/NAD^+ ratio, with total inhibition of measured in vitro activity at an NADH/NAD^+ ratio lower than 0.03 (Fig. 3).

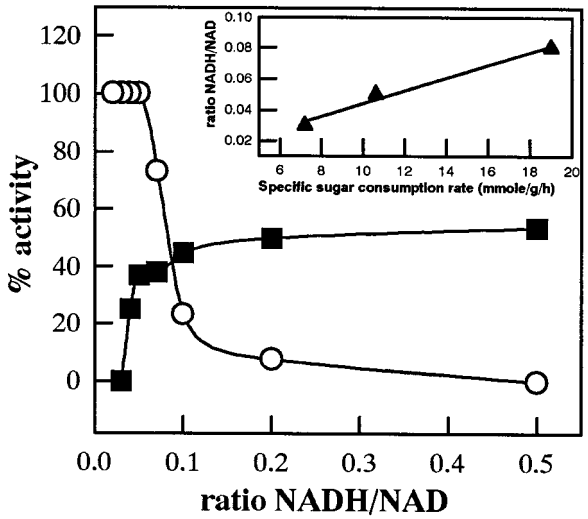

FIG. 3. Effect of the NADH/NAD^+ ratio on the in vitro specific activity of GAP dehydrogenase (○) and LDH (■) measured in extracts prepared from exponentially growing cells. Identical profiles were obtained in extracts prepared with glucose- or lactose-grown cells. The insert shows how the NADH/NAD^+ ratio (▲) varied relative to the exponential growth rate obtained on glucose, galactose, and lactose.

TABLE 3. Modeling of the specific activities of LDH and GAP dehydrogenase, taking into account the effect of the NADH/NAD^+ ratio, on each substrate tested

Enzyme	Activity (nmol/min/mg of dry cells)[a]	Flux	Activity/flux	% Inhibition	Corrected activity (nmol/min/mg of dry cells)	Corrected activity/flux	Predicted substrate concn (mM)
Glucose-grown cells							
LDH	4,274	484	9	52	2,051	4	1
GAPDH	1,261	581	2	50	630	1	>3
Galactose-grown cells							
LDH	2,210	202	11	65	773	4	1
GAPDH	1,813	360	5	0	1,813	5	0.1
Lactose-grown cells							
LDH	2,976	6	496	99	30	5	1
GAPDH	1,090	195	5	0	1,090	5	0.1

[a] Measured relative to protein content of extracts, taking into account the experimentally determined soluble protein content of the cells.

(ii) PFL. The K_m of PFL for pyruvate was found to be approximately 1 mM. DHAP and GAP inhibited activity to equal extents, with inhibition constants (K_I) of 0.2 mM, suggesting a strict control of in vivo activity in response to the triose phosphate pool variations.

(iii) GAP dehydrogenase. The maximum rate of GAP dehydrogenase activity in crude extracts was 1,800 nmol/min/mg, with an apparent K_m for GAP of 0.3 mM. The enzyme was strongly inhibited by an NADH/NAD^+ ratio above 0.05, though no inhibition was found at lower ratios (Fig. 3).

DISCUSSION

The shift from homolactic to mixed-acid fermentation was directly correlated to the flux through glycolysis as estimated from the specific rates of sugar consumption. The gain in ATP associated with the increased acetate production during mixed-acid fermentation was sufficient to enable the growth on lactose to establish at a value similar to that on galactose. In both cases, the efficiency of biomass synthesis relative to ATP production (Y_{ATP}) remained constant at approximately 10 g of biomass per mol of ATP, a value typical of that obtained for *L. lactis* (1). Thus, under growth conditions in which the glycolytic flux is low, presumably due to inefficient sugar uptake and/or the lack of the tagatose pathway, the cell derives some benefit from switching metabolism toward mixed-acid fermentation despite the fact that the products of this pathway are more growth inhibitory than lactate (12). Of course, in many environmental niches, these products will be continuously exploited by other members of the microbial community, and hence mixed-acid fermentation might be considered the more efficient from an energetic viewpoint.

Even if the advantages to the organism of such a metabolic shift can be identified, the mechanism provoking this shift has never been adequately explained. The level of gene expression, as measured by the concentration of active enzyme within the cell, consolidates the change in shift but cannot alone explain the phenomenon. The controlling influence of triose phosphates on PFL activity (19, 23) during homolactic fermentation was confirmed at concentrations coherent with the level of in vivo metabolite pools. During glucose fermentation, triose phosphate pools were sufficiently high to ensure virtually complete inhibition of PFL activity, while during mixed-acid conditions, the pool concentrations were estimated (see below) to be below the K_I value of the enzyme, and hence control over this enzyme would have been relaxed.

The fact that the FBP concentration varies significantly in response to pathway flux and is known to be an essential activator of LDH has led to the hypothesis that the homolactic fermentation is controlled by the FBP pool (21, 22, 27). However, in this study the in vitro analysis of LDH activation by FBP and the absence of any significant antagonistic effect of P_i is not consistent with any in vivo role of these factors. The pool of FBP was always sufficient to ensure full activation of LDH, even on lactose, in which no significant flux toward lactate occurred. Inhibition of LDH by physiological PEP concentrations was also too weak to be effective in vivo. However, the control exerted by the NADH/NAD^+ ratio on LDH would explain the low flux through this enzyme during growth on lactose. The specific activity of LDH has been modeled according to the kinetic constants obtained in this study, taking into account the ambient in vivo environment during growth on each sugar, and compared with the measured flux through the pathway. A large apparent excess of enzyme was found (activity 9-, 11-, and 500-fold higher than the flux in glucose-, galactose-, and lactose-grown cells, respectively), but once the inhibitory effect of the NADH/NAD^+ ratio is taken into account, this apparent excess falls to a factor of 4 to 5 (Table 3). One can easily calculate, from the kinetic characteristics of the LDH and the apparent overcapacity of the enzyme, the substrate concentration which would support adequate flux. A pyruvate pool of approximately 1 mM, a value consistent with the detection threshold of our assay procedure, would be sufficient to support the in vivo flux through LDH. Such a low pyruvate pool might also explain why no significant flux was diverted to acetoin production via acetolactate synthase, whose affinity for pyruvate is extremely low (18).

A similar though opposing control by NADH/NAD^+ exists for GAP dehydrogenase. This coordinated regulation of glycolysis and fermentation pathways is a logical manner in which the cell can regulate pathway flux via coenzyme balances which are extremely sensitive to even minor perturbations of metabolism. Previous work has suggested a controlling influence of GAP dehydrogenase (14), but we would suggest that this enzyme is likely to have such a role only when the glycolytic flux is high. Under such conditions, the metabolism, as portrayed by metabolite pool concentrations, takes place at the level of GAP dehydrogenase: high concentrations of pools upstream of this reaction (FBP, GAP, and DHAP) and low concentrations of pools downstream. In the case of lactose metabolism, in which the pathway flux is most probably limited principally by the sugar transport capacity in this strain, GAP dehydrogenase would be expected to have a considerably diminished influence

on pathway flux. Modeling of the specific activities of GAP dehydrogenase, taking into account the effect of NADH/NAD^+ inhibition in a similar manner as for LDH, shows that the enzyme is present at levels which are fivefold higher than flux during growth on galactose and lactose but is just adequate to satisfy the flux during growth on glucose (Table 3). Thus, the intracellular GAP pool concentration will be saturating during growth on glucose (i.e., >3 mM) but can be predicted to be in the order of 0.1 mM during growth on either galactose or lactose.

The sensitivity of GAP dehydrogenase to the NADH/NAD^+ ratio has also been demonstrated in other gram-positive bacteria such as *Clostridium acetobutylicum* (8) and *Corynebacterium glutamicum* (7), though in both cases this led to overflow of metabolites derived from trioses rather than a profound modulation of pyruvate metabolism. The role of the NADH/NAD^+ ratio has previously been shown to modify pyruvate metabolism principally via the inhibition of pyruvate dehydrogenase activity in *Enterococcus faecalis* (17). In this study, it has been demonstrated that coenzyme equilibria have a coordinated effect on pathway flux at the level of various dehydrogenase reactions.

Other sites of metabolic control will certainly contribute to the regulation of sugar catabolism. One such site may well be localized at the conversion of PEP to pyruvate, catalyzed by both the PTS uptake system and pyruvate kinase. It is interesting that a high pool of PEP, suggesting a possible rate-limiting reaction, was seen on glucose but not on lactose, for which no PTS activity is present in this bacterium. The high pool of PEP on glucose is particularly surprising since the major activators of pyruvate kinase (sugar-phosphates) were all present at relatively high intracellular concentrations. More detailed biochemical study of this step will no doubt clarify the regulatory mechanisms involved.

The genes for many of the enzymes of sugar fermentation in *L. lactis* are now available, and the simplicity of this fermentation pathway as well as the economic importance of the lactic acid bacteria are such that this biological system is probably one of the most appropriate for the study of metabolic control of glycolysis.

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